with 0.01 percent H_2O_2. The sections were then rinsed and stained with 0.01 percent Evan's blue for 2 minutes, mounted in a mixture of glycerol and PBS (1:1 by volume) and examined. For cytochemical controls see (*11*).

11. Perfusion fixation was done as described in (*10*) but with 1 percent glutaraldehyde in PBS being used as the fixative for 2 hours at room temperature. Free aldehyde groups were blocked by subsequent incubation of small tissue fragments in 0.5*M* NH_4Cl in PBS for 2 hours. The fragments were rinsed in PBS, dehydrated in ethanol, and embedded in Epon 812. Thin sections (400 to 600 μm) were placed on parlodion-carbon coated nickel grids (200 mesh) and processed by the pAg technique as follows. The grids were placed, sections downward, on a droplet of 1 percent ovalbumin in PBS for 5 minutes and then transferred to a droplet of the antiserum against chicken CaBP (diluted 1/100) and incubated for 16 hours at 4°C. The grids were rinsed twice with PBS and reacted with the pAg solution for 1 hour at room temperature. After two further rinses in PBS and a final rinse in distilled water, the thin sections were counterstained with uranyl acetate and lead citrate. Controls for cytochemical specificity included (i) the replacement of the diluted antiserum by normal rabbit serum or by antiserum previously adsorbed with purified chicken duodenal CaBP (400 μg of CaBP per milliliter of undiluted antiserum), (ii) incubation of the sections with nonlabeled protein A (0.1 mg/ml) between the antiserum and pAg incubation step, and (iii) staining for endogenous peroxidase activity. The thin sections were examined with a Philips EM 300. For further details see J. Roth, M. Bendayan, L. Orci, *J. Histochem. Cytochem.* **26**, 1074 (1978); *ibid.* **28**, 55 (1980).
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23. We thank M. Amherdt for help with the quantitative analysis, D. Brown for helpful discussions, and F. Fichard, M. Sidler-Ansermet, and I. Bernard for technical assistance. This work was supported in Geneva by Swiss National Science Foundation grant 3.668.80, and in Riverside by PHS grants AM-09012 and AM-14,750. Reprint requests should be addressed to J.R.

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Sulfhydryl Compounds May Mediate Gastric Cytoprotection

Abstract. *Ethanol induces hemorrhagic gastric erosions and causes a dose-dependent decrease in the concentration of nonprotein sulfhydryl compounds in rat gastric mucosa. Sulfhydryl-containing drugs protect rats from ethanol-induced gastric erosions, whereas sulfhydryl blocking agents counteract the mucosal cytoprotective effect of prostaglandin $F_{2\beta}$. These observations suggest that endogenous nonprotein sulfhydryls may mediate prostaglandin-induced gastric cytoprotection and that sulfhydryl drugs may have potential for preventing or treating hemorrhagic gastric erosions.*

Severe stress or ingestion of ethanol, aspirin, and other nonsteroidal anti-inflammatory agents are predisposing causes of acute hemorrhagic gastric erosions in humans (*1*). Whereas chronic ulcers with inflammation usually occur in the antral or duodenal mucosa and penetrate the mucosal muscle to involve deeper layers, hemorrhagic gastric erosions primarily involve the acid-producing portion of the stomach, are limited to the mucosa, and cause little or no surrounding inflammation (*2*). Morphologically identical superficial erosions can be consistently induced in the acid-secreting glandular stomach of laboratory rodents by intragastric administration of ethanol and aspirin (*3*).

Erosions of the rat gastric mucosa induced by noxious substances (such as ethanol and aspirin) are fewer and less severe if prostaglandins are first administered. This effect, termed cytoprotection (*4*), is shared by virtually all prostaglandins (types A, B, C, D, E, F, and I); inhibition of acid secretion is not involved. The dose of prostaglandins required for cytoprotection is much smaller than that required for inhibition of acid secretion. Furthermore, some prostaglandins that do not inhibit acid secretion (such as prostaglandin $F_{2\beta}$) induce cytoprotection (*4*).

The mechanism of cytoprotection is unknown (*4, 5*). Gastric mucus glycoproteins may play a role (*4*). Tissue injury produced by noxious agents may result in the accumulation of toxic free radicals in mucosal cells. The gastric mucosa contains unusually high concentrations of reduced glutathione (*6*), the major component of the endogenous nonprotein sulfhydryl pool. Thiols such as reduced glutathione are able to bind reactive free radicals (*7*) and may influence the physical properties of mucus, since its subunits are joined by disulfide bridges (*8*). Boyd *et al.* (*6*) reported that diethylmaleate, an agent that markedly depletes gastric glutathione, causes severe gastric ulceration, suggesting a possible modulatory role for glutathione in ulcerogenesis. We performed a series of experiments to determine the effects of ethanol, thiols, and sulfhydryl blocking agents on mucosal nonprotein sulfhydryls and on gastric cytoprotection. We found that ethanol lowers the concentration of nonprotein sulfhydryls in gastric mucosa, that thiols induce gastric cytoprotection while increasing nonprotein sulfhydryl levels, and that sulfhydryl blocking agents prevent the cytoprotective effect of prostaglandin $F_{2\beta}$ and decrease nonprotein sulfhydryl levels.

In the first experiment, female Sprague-Dawley rats (150 to 200 g) on a diet of Purina Lab Chow and tap water were fasted overnight, given 1 ml of water or various concentrations of ethanol by gavage (Fig. 1), and killed 5 minutes later (*9*). (This interval was selected since major mucosal lesions do not appear during the first 5 minutes after exposure to ethanol.) Mucosa from glandular stomach and part of the left lobe of the liver were rapidly removed, weighed, and frozen for subsequent homogenization and measurement of protein and nonprotein sulfhydryl concentrations (*10*).

The concentration of nonprotein sulfhydryls in the gastric mucosa decreased significantly after only 5 minutes of exposure to 50 or 70 percent ethanol (Fig. 1). Exposure to 100 percent ethanol caused even more severe depletion. In contrast, liver nonprotein sulfhydryls (Fig. 1) and protein sulfhydryls in liver and gastric mucosa were unchanged 5 minutes after intragastric administration of ethanol at each of the test concentrations.

Gastric washings collected before the administration of ethanol contained only trace amounts of nonprotein sulfhydryls; 5 minutes after 100 percent ethanol was administered, the amount of nonprotein sulfhydryls removed from the gastric lumen more than doubled (235 percent). This may represent leakage of nonprotein sulfhydryls from remaining mucosal cells or nonprotein sulfhydryls present in mucosal cells that were exfoliated into the lumen as a result of ethanol administration.

In the second study, rats received prostaglandin $F_{2\beta}$ (0.05 mg per 100 g, by gavage) or one of several sulfhydryl-containing drugs including dimercaprol

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(3 mg, intramuscularly) cysteamine hydrochloride (30 mg, by gavage), 2,3-dimercaptosuccinic acid (60 mg, by gavage), or penicillamine (60 mg, by gavage). After 30 minutes absolute ethanol (1 ml) was administered intragastrically by gavage; the animals were killed 1 hour later. Other rats received one of two sulfhydryl blockers, iodoacetamide (10 mg, subcutaneously) or *N*-ethylmaleimide (5 mg, subcutaneously), alone or 10 minutes after intragastric administration of prostaglandin $F_{2\beta}$. The rats receiving sulfhydryl blockers alone were given ethanol after 20 minutes and killed 1 hour later. The others were given ethanol after 30 minutes and killed 1 hour later. The stomach was removed and opened along the greater curvature, and the severity of hemorrhagic erosions in the acid-secreting glandular mucosa was assessed on a scale of 0 to 3. As documented in Table 1, the sulfhydryl drugs, like prostaglandin $F_{2\beta}$, significantly reduced the gastric erosions caused by ethanol. On the other hand, the sulfhydryl blockers induced no cytoprotection and also counteracted the cytoprotective effect of prostaglandin $F_{2\beta}$. These doses of sulfhydryl drugs or blockers significantly altered the concentration of nonprotein sulfhydryls in gastric mucosa. The concentration of nonprotein sulfhydryls was $53.1 \pm 2.8 \times 10^{-5}$ mole per gram of tissue (wet weight) in control stomachs and $85.6 \pm 6.6 \times 10^{-5}$ ($P < .01$, Student's *t*-test) and $98.0 \pm 10.9 \times 10^{-5}$ ($P < .001$) mole per gram 30 minutes after administration of cysteamine and penicillamine, respectively. In contrast, 20 minutes after injection of iodoacetemide or *N*-ethylmaleimide, nonprotein sulfhydryls decreased to $35.8 \pm 2.2 \times 10^{-5}$ ($P < .005$) and $40.5 \pm 2.5 \times 10^{-5}$ ($P < .05$) mole per gram, respectively. Furthermore, administration of cysteamine (1, 5, 10, or 30 mg by gavage, 30 minutes before ethanol was given or before the rats were killed) caused a dose-dependent increase in nonprotein sulfhydryls and a decrease in ethanol-caused mucosal erosions (Table 2) (*11*). These data are additional evidence of the importance of nonprotein sulfhydryls in gastric mucosal cytoprotection.

These findings indicate that ethanol-induced erosions in rat gastric mucosa are preceded by a substantial decrease in the concentration of nonprotein sulfhydryls, of which the major fraction is reduced glutathione (*6*, *7*). Moreover, sulfhydryl-containing drugs exert a gastric cytoprotective effect similar to that of prostaglandins. In earlier studies doses of cysteamine comparable to those used in this study also increased the concentration of nonprotein sulfhydryls in the gastric mucosa (*12*). Thus both endogenous and exogenous nonprotein sulfhydryls may provide gastric cytoprotection when present in the gastric mucosa in adequate amounts. The inability of prostaglandin $F_{2\beta}$ to exert its cytoprotective effect in the presence of sulfhydryl blockers suggests that endogenous sulfhydryls facilitate this beneficial action of prostaglandins.

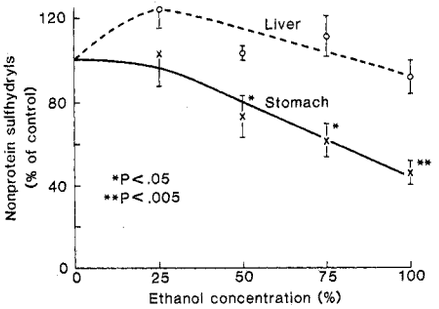

Fig. 1. Effect of ethanol on the concentration of nonprotein sulfhydryls in rat gastric mucosa and liver. Each point represents the mean (± standard error) for six to eight rats. Animals were killed 5 minutes after receiving ethanol (*10*). The results were analyzed by Student's *t*-test.

Although nonprotein sulfhydryls appear to be involved in gastric cytoprotection, their mechanism of action remains unknown. Because of their exposed sulfhydryl groups, nonprotein sulfhydryls bind a variety of electrophilic radicals and metabolites (*7*) that may be damaging to normal cells. If radicals or metabolites with an affinity for sulfhydryl groups accumulate during ethanol-induced gastric mucosal injury, nonprotein sulfhydryls, especially glutathione, might reduce their concentration and thus facilitate cytoprotection. Interestingly, the concentration of nonprotein thiols is high in organs that are highly exposed to noxious chemicals (*6*, *12*). Furthermore, reduced nonprotein sulfhydryl compounds may influence the structure of gastric mucus or gastric

Table 1. Effect of sulfhydryl drugs and blockers on gastric cytoprotection following ethanol administration.

Group	Pretreatment	Gastric erosion						
		Scale*				*N*	Mean	*P*†
		0	1	2	3			
	Controls							
1	None	0	7	16	3	26	1.9	
2	Prostaglandin $F_{2\beta}$	4	7	1	0	12	0.7	.0003
	Sulfhydryl drugs							
3	Dimercaprol	5	8	1	0	14	0.7	<.0001
4	Cysteamine	10	2	0	0	12	0.2	<.0001
5	Dimercaptosuccinic acid	6	3	0	0	9	0.3	<.0001
6	Penicillamine	3	4	1	0	8	0.8	.0025
	Sulfhydryl blockers							
7	Iodoacetamide	0	0	2	1	3	2.3	.2519
8	*N*-Ethylmaleimide	0	0	1	5	6	2.8	.0038
9	Iodoacetamide + prostaglandin $F_{2\beta}$	0	1	5	0	6	1.8	.9615
10	*N*-Ethylmaleimide + prostaglandin $F_{2\beta}$	0	0	1	6	7	2.9	.0016

*Categories are as follows: 0 = normal; 1 = one to four small petechiae; 2 = five or more petechiae or hemorrhagic streaks up to 4 mm; 3 = erosions longer than 5 mm or confluent hemorrhages. †Results for the treatment groups were compared against those for the control group with a two-sided Wilcoxon rank-sum test incorporating a large sample approximation (*14*). Controlling for an overall type 1 error rate of .05, we used $\alpha = .05/9 = .0056$ for each comparison. This is a conservative criterion; the probability of a type 1 error is somewhat lower than .05.

Table 2. Relation between severity of ethanol-induced gastric erosions and concentration of nonprotein sulfhydryls in the mucosa.

Group	Pretreatment	Nonprotein sulfhydryls ($\times 10^{-5}$ mole/g)	Gastric erosion*						
			Scale				*N*	Mean	*P*†
			0	1	2	3			
1	None	76.1 ± 4.9	0	0	9	3	12	2.3	
2	Cysteamine, 1 mg	69.6 ± 10.9	0	1	5	0	6	1.8	.2234
3	Cysteamine, 5 mg	103.8 ± 26.7	0	4	2	0	6	1.3	.0131
4	Cysteamine, 10 mg	127.0 ± 25.2‡	2	2	2	0	6	1.0	.0125
5	Cysteamine, 30 mg	253.1 ± 23.0‡	6	2	1	0	9	0.4	.0005

*These rats were given 1 ml of 100 percent ethanol 30 minutes after receiving cysteamine. They were killed 1 hour later. †Two-sided Wilcoxon rank-sum test with large sample approximation. ‡$P < .005$, Student's *t*-test.

membrane glycoprotein. Reduction of the disulfide bridges in gastric mucus markedly alters its physical state by converting it from a water-insoluble gel to a water-soluble form (*8*). Derivatives of the sulfhydryl-containing amino acid cysteine have been shown to liquefy mucus (*13*). However, if mucus plays a role in gastric cytoprotection, its optimal form, gel or liquid, is not known. Another possibility is that tissue thiols mediate cytoprotection by enhancing prostaglandin synthesis or inhibiting prostaglandin breakdown.

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9. Immediately after death only mild injury of the gastric mucosa was evident on gross inspection; the maximal effect was seen 15 to 30 minutes later.
10. Since there is diurnal variation in the concentration of nonprotein sulfhydryls in tissue, all animals were killed between 9 and 11 a.m. Mucosa in the glandular stomach was washed with saline and then removed by scraping with a blunt knife. Protein and nonprotein sulfhydryls were measured as described by J. Sedlack and R. Lindsay [*Anal. Biochem.* **24**, 192 (1968)]. Gastric mucosa and liver samples were placed in 40 volumes of 0.2*M* aqueous EDTA and homogenized at 50 rev/min for 6 seconds. Ellman's reagent was added and the concentration of sulfhydryls was determined by measuring the absorbance at 412 nm. All assays were performed in duplicate. Results were calculated as concentration of protein and nonprotein sulfhydryls per gram of tissue.
11. The gastric mucosal concentration of protein and nonprotein sulfhydryls and disulfides was measured by a highly sensitive method modified from A. F. S. A. Habeeb [*Anal. Biochem.* **56**, 60 (1973)] and J. Isaacs and F. Binkely [*Biochim. Biophys. Acta* **497**, 192 (1977)]. Because of the sensitivity of this method, the nonprotein sulfhydryl levels are slightly higher than those obtained by the procedure of Sedlack and Lindsay (*10*).
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Gonadal Hormones Induce Dendritic Growth in the Adult Avian Brain

Abstract. *Ovariectomized adult female canaries were treated with physiological doses of testosterone, dihydrotestosterone, or estradiol. Singing, which is typical of males, occurred in the testosterone-treated birds but not in any of the other birds. The effect of these hormones was assessed on dendrites from a class of neurons in the nucleus robustus archistriatalis (RA), a forebrain nucleus for song control. The RA neurons of the testosterone-treated birds had dendritic trees resembling those of intact males. The RA neurons of the estradiol- and dihydrotestosterone-treated birds resembled those of intact females. All hormone-treated groups had dendrites that were significantly longer than those of untreated ovariectomized females. Thus gonadal hormones induce dendritic growth in the adult avian brain.*

Plasticity in the central nervous system is often thought to be a property of the very young brain. In the development of sexually dimorphic neural systems, the role of steroid hormones is believed to be most powerful at early stages (*1*). We here report experiments that show that steroid hormones can also play a powerful role in inducing neural plasticity in the adult brain.

The canary song system offers the possibility of relating differences in the adult hormonal milieu to altered neuronal organization and changed behavioral capabilities. Several discrete brain nuclei are responsible for song control in this species (*2*). Normally, males sing and females do not. This behavioral dimorphism is reflected in the volume of brain song control nuclei (*3*). Sexual dimorphism is also present at the microanatomical level. We have recently shown that the length and distribution of dendrites of cells in a telencephalic song control nucleus, the nucleus robustus archistriatalis (RA), are highly dimorphic in adult male and female canaries (*4*, *5*). The overall volume of RA increases after testosterone administration to adult females (*6*), a treatment that also induces females to sing in a male-like manner (*6*).

The present experiment was planned to assess neuronal consequences of administering gonadal hormones to ovariectomized adult female canaries, and to relate these effects to the microanatomical configuration seen in intact adult male and female canaries. We studied in detail a neuron type with long, sinuous, spiny dendrites within RA. These cells, which stain reliably and can easily be distinguished from other cell classes within RA, were the object of our previous work assessing neuroanatomical sex differences in intact canaries (*5*).

Thirty-six female canaries were ovariectomized between 12 and 16 days after hatching. When they were 11 months of age we implanted in each bird a Silastic tube containing testosterone, dihydrotestosterone (DHT), estradiol (E), or nothing (ovariectomized control) (*7*). Three weeks later we made tape recordings of the birds that were singing. At 4 weeks after implantation the birds were killed and their brains were removed and stained by a rapid Golgi procedure (*5*). The brains were sliced at 100 μm in the plane of Stokes *et al.* (*8*). Cells of the type in which we were interested were found in RA in the brains of six testosterone-treated birds and seven each of the E- and DHT-treated birds and the ovariectomized controls. A computer-microscope system was used to record the coordinates of at least ten cells each from the right and left hemispheres of the brains in each group. As in earlier studies comparing cells of this class from male and female canaries (*5*), we described all dendritic branches, including those in adjacent tissue sections, using three-dimensional coordinates. We then analyzed the data by measuring dendritic branch lengths at different orders of branching from the cell body and by the Sholl concentric sphere analysis (*9*).

Each of the testosterone-treated ovariectomized females sang vigorously. No other ovariectomized female sang. However, in studies of hormone dosages we observed that six intact females treated with DHT-filled Silastic tubes like those used in the present study also sang vigorously. Thus testosterone, or a combination of its metabolites E and DHT, seems

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